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Partial retrotransposon-like DNA sequence in the genomic clone of *Aspergillus flavus*, pAF28

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A genomic clone of the aflatoxin-producing fungus *Aspergillus flavus*, designated pAF28, has been used as a probe for Southern blot fingerprinting of fungal strains. A large number of *A. flavus* strains isolated from corn fields and tree-nut orchards can be distinguished because the DNA fingerprint patterns are highly polymorphic. We have completed the sequencing of a 6355 bp insert in pAF28. The sequence features motifs and open reading frames characteristic of transposable elements of the *gypsy* class. We have named this new element *AfRTL-1*, for *A. flavus* retrotransposon-like DNA.

INTRODUCTION

Aspergillus flavus is the most common aflatoxin-producing species on corn, cotton, peanuts and tree nuts (Diener *et al.* 1987). Aflatoxin is a potent hepatoxin and carcinogen that poses a serious food safety hazard to both humans and animals. The presence of aflatoxin reduces the quality and value of infected agricultural products, directly affecting the economic return to both growers and processors and posing a health hazard to consumers. The genomic fragment pAF28, previously isolated from *A. flavus* strain NRRL 6541 (McAlpin & Mannarelli 1995), not only hybridizes strongly to DNA of *A. flavus* and *A. oryzae* but also hybridizes with lower intensity to DNA of other species closely related to the *Aspergillus* section *Flavi* including *A. parasiticus*, and *A. sojae* (McAlpin & Mannarelli 1995, McAlpin, Wicklow & Horn 2002). pAF28 has been used extensively as a hybridization probe on Southern blots with restriction fragment length polymorphisms (RFLP) that distinguish between numerous genotypes of *A. flavus* isolated from corn, peanuts, pistachios and almond (McAlpin & Mannarelli 1995, Hua & McAlpin 2001, McAlpin *et al.* 2002). The ability of pAF28 to distinguish strains of *A. flavus* belonging to different characterized vegetative compatibility groups (Papa 1986, McAlpin & Mannarelli 1995, McAlpin *et al.* 2002) indicates its further utility. In this study, we demonstrate that the genomic insert of pAF28 carries a partial retrotransposon-like element homologous to the *gypsy*-class retrotransposon *MAGGY*, originally isolated from *Magnaporthe grisea* (Farman *et al.* 1996), and retrotransposons from several other fungi (McHale *et al.* 1992, Dobinson, Harris & Hamer 1993, Anaya & Roncero 1995, Hamann, Feller & Osiewacz 2000, Kaneko, Tanaka & Tsuge 2000, Murata & Yamada 2000, Zhu & Oudemans 2000).

MATERIALS AND METHODS

Subcloning pAF28 DNA

The construction and propagation of plasmid DNA was performed using standard protocols (Sambrook, Fritsch & Maniatis 1989). DNA fragments generated by double digestion of pAF28 with restriction endonuclease *Eco*RI and *Bam*HI; *Eco*RI and *Pst*I; *Eco*RI and *Hin*dIII; *Eco*RI and *Sph*I; *Eco*RI and *Sac*I; *Eco*RI and *Sma*I; *Eco*RI and *Kpn*I were subcloned into pUC19, transformed into competent *Escherichia coli* strain JM109 (Promega, Madison, WI) and grown on LB (Lauria Broth) agar plates containing ampicillin (50 μg ml^{-1}). Plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).

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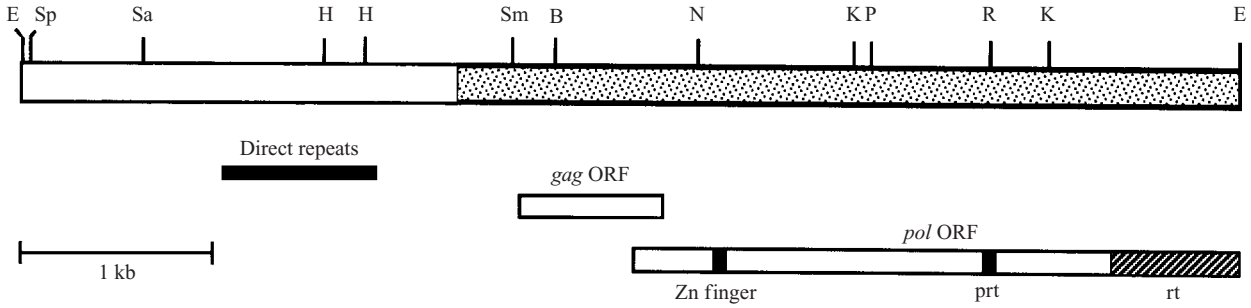

Fig. 1. Restriction map of the 6.36 kb *AfRTL-1* DNA fragment from pAF28. The stippled portion indicates the sequence deposited in GenBank (accession no. AF362957). Restriction sites are indicated as E, *Eco*RI; Sp, *Sph*I; Sa, *Sac*I; H, *Hin*dIII; Sm, *Sma*I; B, *Bam*HI; N, *Nco*I; K, *Kpn*I; P, *Pst*I; R, *Eco*RV. Restriction sites shown in boldface type were used to generate subclones for sequencing. The black bar denotes the location of direct repeats. Open bars show the putative *gag* and *pol* open reading frames (ORF). The regions of the *pol* ORF encoding an RNA binding zinc finger (Zn finger), protease (prt) and reverse transcriptase (rt) domains are shaded.

DNA sequencing

The nucleotide sequence of a 6.3 kb *Eco*RI genomic fragment in pAF28 was determined using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Sequencer Kit and ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA). The fragment was sequenced bidirectionally using the following primers (OPERON Technologies, Alameda, CA):

M13F(-20), 5′-TGTAAAACGACGGCCAGT
P3-F21, 5′-CAAGAATGGCGCTGGCTACAC
P4-F24, 5′-TTTAGATAGAGCGCGTTTAGAAGC
P2-F19, 5′-GCCTGGCCCTTTGGACTCG
RB, 5′-CAACAACGCCAAAGAAAAG
P6-F24, 5′-AGGACGCAATCGGAAAAGTGAAAC
P1-F21, 5′-GGTGGACGGCCCTGATAATAC
RA, 5′-GATTGGAAGCAACGGAC
P5-F24, 5′-ATCGAAGGACGGCAAAAGGAAAAC
M13R, 5′-CAGGAAACAGCTATGACC
P5-R24, 5′-AATGAGCGGTAGTGGGTGTCTGTC
FD, 5′-CCTGCTGGACTTTTCG
P1-R21, 5′-TCGCGCTGGTTTTCCGTTGAC
FA, 5′-CGGTGGTACAGTATGCTC
P6-R20, 5′-TCCCGGTGGTACAGATGCTC
P2-R20, 5′-CAATTCTCGCGTGGTGTTCG
FB, 5′-GGCATCGTCATCTATCG
P4-R23, 5′-AATTGCGGTGCGTAGCGTCGTAT
P3-R24, 5′-CTTCTAAACGCGCTCTATCTAAAT

Sequence data were compiled into contigs using the ABI Data Collection software (version 1.0.2) and AutoAssembler™ (version 1.4.0). A 6355 bp DNA sequence containing the full sequence of pAF28 has been submitted to GenBank (accession no. AF362957).

Computer-based identity searches and alignments

Deduced open reading frames in *AfRTL-1* were compared to GenBank database entries using BlastX (Altschul *et al.* 1997). Selected segments of the *AfRTL-1* deduced polypeptides were aligned to fungal retrotransposon polypeptides using CLUSTAL W 1.8 (Jeanmougin *et al.* 1998, http://searchlauncher.bcm.tmc.edu:9331/multi-align/multi-align.html). The Multiple Sequence Alignment Editor and Shading Utility 2.5 program (Nicholas & Nicholas 1997) was used to further identify conserved portions of the Gag and Pol polypeptides. The *AfRTL-1* DNA sequence was analyzed for enhancer elements and LTR core elements using PrimerSelect 3.11 (DNAStar, Madison, WI), and for direct repeats using the DotPlot subroutine of MegAlign 3.18 (DNAStar).

RESULTS AND DISCUSSION

The AfRTL-1 *DNA sequence*

McAlpin & Mannarelli (1995) previously identified and cloned a 6.3 kb genomic DNA fragment, designated as pAF28 from *Aspergillus flavus* var. *flavus* NRRL 6541. The pAF28 fragment is 6355 bp in length. A restriction map derived from our new sequence data compared closely with that of McAlpin & Mannarelli (1995), with the exception of an additional *Hin*dIII site located about 200 nucleotides upstream of the one reported previously (Fig. 1). This cleavage site had not been detected by conventional mapping, possibly because it generated a relatively small fragment.

AfRTL-1 *encodes ORFs common to fungal* gypsy-*class retrotransposons*

Our sequence data revealed that pAF28 contains several features common to retrotransposon-like elements. Two major overlapping open reading frames (ORFs) of 240 and at least 980 amino acids, respectively, occurred within the 4.5 kb *Sma*I–*Eco*RI fragment of *AfRTL-1* (Fig. 1). The first ORF showed identity to polypeptides encoded by *gag* genes of the *gypsy* class of fungal retrotransposons, including *CfT-I* from *Cladosporium fulvum* (McHale *et al.* 1992), *skippy* from *Fusarium oxysporum* (Anaya & Roncero 1995), and *Cgret* from *Colletotrichum gloeosporioides* (Zhu & Oudemans

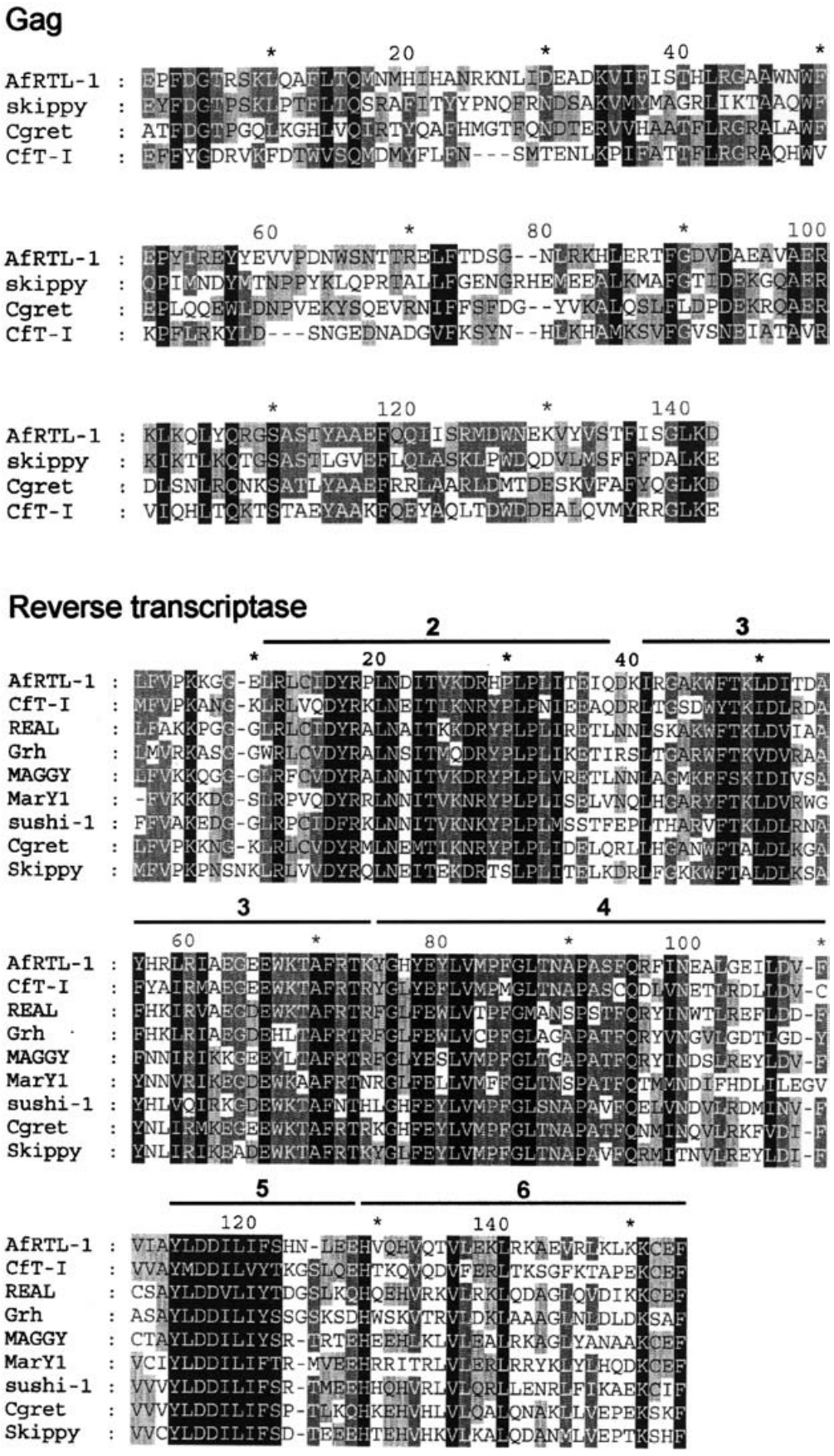

Fig. 2. Alignments of portions of the deduced Gag (upper panel) and reverse transcriptase (lower panel) polypeptides of *AfRTL-1* with those from fungal and *Fugu*-associated retrotransposons. Black shaded areas indicate regions of highly conserved amino acids. Conserved reverse transcriptase domains (Xiong & Eickbush 1990) are located by bars over the alignment as follows: domain 2 (11–39); domain 3 (41–74); domain 4 (75–110); domain 5 (114–128); domain 6 (129–154).

Table 1. Comparison of *AfRTL-1* with retrotransposons of the *gypsy* class from other fungal species.

Element	GenBank nos.	Length (bp)	*gag* ORF	*pol* ORF	*gag* RNA BD[a]	Protease domain	RTase domain 5
AfRTL-1	AF362957	Unknown	240	>980	CYNCGRAGHMSKDC	AMIDSGATNNF	VIAYLDDILIFS
CfT-I	S23569, AF051915	6968	639	1045	CYGCGKPGHIARDC	AMIDSGASGNF	VVAYMDDILVYT
skippy	S60178, S60179	7846	854	1296	CYNCGKKGHYEREC	ALVDSGADMNF	VVCYLDDILIFS
MAGGY	L35053, T18348	5638	457	1260	CYRCGSQEHFVAKC	ALTDCGAEGKCF	CTAYLDDILIYS
REAL	AB025309, BAA89272	6046	406	~1300	CYSCGKPGHIARDC	ALVDSGCLCYSL	CSAYLDDVLIYT
marY1	AB028236	6046	352+111[b]	1057	CYRCGEPGHRAGAC	Unknown	VCIYLDDILIFT
Cgret	AAG24791, AAG24792	7916	–	–	CFNCNQKGHLAYEC	ALIDSGSEGDF	VVVYLDDILIFS
Grasshopper	M77661, M77662	8000	–	1107	CLRCGNSGHQVADC	AVQDSGCECYAA	ASAYLDDILIYS

[a] *gag* Zn finger RNA binding domain.
[b] *gag* and *prt* have separate open reading frames.

2000). The most highly conserved portions of the Gag polypeptides are shown in Fig. 2. The deduced *gag* ORF of *AfRTL-1* was shorter than those from other sources (Table 1) due to the presence of a stop codon. However, a GAAAAG sequence beginning at nucleotide 5465 (in AF362957) could serve as the site for a −1 frame shift, which would extend the *gag* ORF. Frameshifts at similar A-rich sites in other *gypsy* retrotransposons are the proposed mechanisms by which the full-length *gag* ORFs are generated (Anaya & Roncero 1995, Kaneko *et al*. 2000). A Zn finger RNA binding domain of the consensus $CX_2CX_4HX_4C$, encoded in the Gag regions of retrotransposons and retroviruses (Covey 1986), was found at the 5′ end of the second ORF (Fig. 1).

The second ORF showed significant amino acid sequence identity to reverse transcriptase domains of a number of fungal retrotransposons of the *gypsy* class (McHale *et al*. 1992, Anaya & Roncero 1995, Farman *et al*. 1996, Hamann *et al*. 2000, Kaneko *et al*. 2000, Murata & Yamada 2000) and to a retrotransposon associated with the pufferfish *Fugu* (Poulter & Butler 1998). These similarities are summarized in Table 1. The most highly conserved portions of the *pol* ORF corresponded to five of the seven reverse transcriptase domains (Xiong & Eickbush 1990) and are shown in Fig. 2. The *pol* ORF in the 6.3 kb *Eco*RI clone was truncated at the region encoding the amino acids CEF, located in a semi-conserved portion of the reverse transcriptase domain (Fig. 2). Therefore, the predicted RNaseH and integrase domains, located downstream of reverse transcriptase in all other retrotransposons of the *gypsy* class, were not present in this clone. However, a protease domain similar to that of *gypsy*-type fungal retrotransposons (Table 1) was located between the Zn finger RNA binding domain and reverse transcriptase domains on the *pol* ORF (Fig. 1).

It is not known whether *AfRTL-1* encodes functional proteins, transcripts of the element or reverse transcriptase activity associated with virus-like particles (e.g. McHale *et al*. 1992). These activities have yet to be demonstrated in *A. flavus* NRRL 6541. Although the ORFs of *AfRTL-1* contain no premature stop codons, frameshifts or interruptions that would result in loss of function, we cannot rule out the possibility that these may occur in the uncloned portion of the element. Silencing of transposons by DNA methylation has been demonstrated in some filamentous fungi (Martienssen & Colot 2001). DNA methylation has recently been detected in *A. flavus* (Gowher *et al*. 2001).

Repeat elements in the 5′ region

The region of *AfRTL-1* upstream of the *gag* ORF contained multiple copies of core enhancer elements and direct repeats. Six *Ty1*/*Neurospora* core enhancer elements (TTCCA) and four *pho80* enhancer elements (TACCA) were located between the *Sph*I and *Sal*I restriction sites (Fig. 1). Longer elements and direct repeats reported in *CfT-I* (McHale *et al*. 1992) and *skippy* (Anaya & Roncero 1995) were not observed upstream of *gag* in *AfRTL-1*. However, four new families of direct repeats were identified in this region. Five copies of TCTATATA, four copies of TAAAAATA, three copies of CTATATAAAA, TTATTTTTTA and TAATATTATT, as well as three imperfect repeats of the consensus GTATCGACGGCAGTCTAGTGTCGACGGCA were scattered throughout a 850 bp region located downstream of the *Sal*I site as indicated in Fig. 1. The significance of these direct repeats is not known at this time; however, the shorter repeats might reflect the AT-rich nature of this region. Owing to the absence of the 3′ portion of the element, we were unable to identify long terminal repeats (LTR) or duplicated target sequences characteristic of *gypsy*-class retrotransposons.

The repeat-rich region of *AfRTL-1* also shows two long stretches of nucleotide sequences (positions 1333–1511 and 1142–1251) having high sequence identity (82–93%) with the transposon *Tao1* from *A. oryzae* (GenBank accession no. AB021710) and a transposon-like element embedded within the amylase gene cluster of *A. oryzae* (Gomi *et al*. 2000). Three DNA regions of 192, 149 and 29 base pairs in length, located about 600 bases upstream of the putative *gag* ORF at nucleotide positions 1881–2404, show 87–93% sequence identity with a transposon-like element in the aflatoxin gene cluster of *A. parasiticus* (Chang & Yu 2002).

Aspergillus *transposons*

The presence of transposon-like elements in the amylase gene cluster of *A. oryzae* (Gomi *et al.* 2000) and the partially duplicated aflatoxin gene cluster of *A. parasiticus* (Chang & Yu 2002) suggests that at least some elements can undergo transposition and affect gene expression. DNA transposon-like elements have also been described in *A. niger* (Glayzer *et al.* 1995, Amutan *et al.* 1996, Nyyssönen *et al.* 1996). DNA transposons differ from retrotransposons in mode of replication and transposition. *Ant1* shows sequence identity to the Tc1/Mariner class of DNA transposable elements (Glayzer *et al.* 1995), whereas *Vader* and *Tan1*, from *A. niger* var. *awamori*, are members of the *Fot1* family of DNA transposons (Amutan *et al.* 1996). Retrotransposable elements have recently been reported in *Aspergillus fumigatus* (Neuveglise *et al.* 1996, Paris & Latge 2001). In contrast to those of the *A. fumigatus* elements, the open reading frames of *AfRTL-1* contain no premature stop codons. pAF28 shows complex hybridization patterns with genomic DNA from the following *Aspergillus* species: *A. sojae*, *A. nomius*, *A. tamarii*, *A. bombycis*, *A. caelatus*, and *A. pseudotamarii* (McAlpin & Mannarelli 1995, McAlpin *et al.* 2002), indicating that multiple copies of *AfRTL-1* or *AfRTL-1*-like sequences occur in many *Aspergillus* species.

Aspergillus *taxa*

As many as 30 bands are detected when pAF28 is hybridized to *Aspergillus flavus* strain NRRL 6541 (McAlpin & Mannarelli 1995). The possibility that *AfRTL-1* exists in other *Aspergillus* species is indicated from the Southern blot data of McAlpin & Mannarelli (1995) in which pAF28 hybridizes with multiple genomic DNA restriction fragments of *A. oryzae* and *A. parasiticus*. Kurtzman *et al.* (1986) found DNA complementarity is 100% between *A. flavus* and *A. oryzae*, 91% between *A. sojae* and *A. parasiticus* and 70% between *A. flavus* and *A. parasiticus*. The data led these researchers to propose that these fungi be designated as varieties of *A. flavus*. Subsequent data obtained by sequence analyses of the rRNA confirm that *A. oryzae*, *A. sojae* and *A. parasiticus* are variants of *A. flavus* (Nikkuni *et al.* 1998, Peterson 2000, Rigo *et al.* 2002). However, it has been suggested that these taxa be retained as separate species due to regulatory concerns and confusion that conspecificity would create in the food industry (Cruickshank & Pitt 1990, Geiser *et al.* 1998).

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